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Assessment of human enteric viruses in cultured and wild bivalve molluscs

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Summary. Standard and real-time reverse transcription-PCR (rRT-PCR) procedures were used to monitor cultured and wild bivalve molluscs from the Ría de Vigo (NW Spain) for the main human enteric RNA viruses, specifically, norovirus (NoV), hepatitis A virus (HAV), astrovirus (AsV), rotavirus (RT), enterovirus (EV), and Aichi virus (AiV). The results showed the presence of at least one enteric virus in 63.4% of the 41 samples analyzed. NoV GII was the most prevalent virus, detected in 53.7% of the samples, while NoV GI, AsV, EV, and RV were found at lower percentages (7.3, 12.2, 12.2, and 4.9%, respectively). In general, samples obtained in the wild were more frequently contaminated than those from cultured (70.6 vs. 58.3%) molluscs and were more readily contaminated with more than one virus. However, NoV GI was detected in similar amounts in cultured and wild samples (6.4×10^2 to 3.3×10^3 RNA copies per gram of digestive tissue) while the concentrations of NoV GII were higher in cultured (from 5.6×10^1 to 1.5×10^4 RNA copies per gram of digestive tissue) than in wild (from 1.3×10^2 to 3.4×10^4 RNA copies per gram of digestive tissue) samples. [**Int Microbiol** 2009; 12(3):145-151]

Keywords: molluscs · enteric viruses · hepatitis A virus · norovirus · viral prevalence · viral quantification · seafood industry

Introduction

Bivalve molluscs growing in coastal areas may be contaminated by human sewage, which can contain more than 100 types of viruses. Due to their filter-feeding nature, bivalve molluscs tend to concentrate these human pathogens and therefore constitute an important vector in the transmission of enteric diseases [3,23]. Viral pathogens have been detected throughout the world in bivalve molluscs from areas with intensive shellfish production or consumption [3,19,26]. Moreover, viruses persist in molluscs for extended periods and, despite technological improvements, depuration does not eliminate viral particles [20,23,32]. These facts contribute to the well-documented human health risk, especially when bivalve molluscs are consumed raw or lightly cooked [2,28]. The periodic outbreaks of bivalve-mollusc-transmitted disease have contributed to a loss of public confidence regarding shellfish safety and, in turn, to important economic losses by the seafood industry [27].

Although only noroviruses (NoV) and hepatitis A virus (HAV) have been clearly implicated in outbreaks linked to shellfish consumption [19,26,33], other enteric viruses, such as enterovirus (EV), astrovirus (AsV), rotavirus (RV) [12,19], and Aichi virus (AiV) [17,37], have been found in shellfish samples. The detection of enteric viruses relies mainly on the use of reverse transcription-PCR (RT-PCR) assays [9], but the low quantity of virus in environmental samples usually requires a time-consuming hybridization step, which enhances both the sensitivity and the specificity of the assays, or sequencing of the obtained amplicons. Recently, real-time reverse transcription-PCR (rRT-PCR) has

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Table 1. Primer sets and probes used for viral detection in this work

Virus	Primer	Probe	Sequence 5´–3´	Fragment size	Reference
NoV GI	QNIF4		CGCTGGATGCGNTTCCAT	98	[7]
	NV1LCR		CCTTAGACGCCATCATCATTTAC		[34]
		NV1Lpr	6-FAM-TGGACAGGAGAYCGCRATCT-6-TAMRA		[34]
NoV GII	QNIF2d		ATGTTCAGRTGGATGAGRTTCTCWGA	95	[21]
	COG2R		TCGACGCCATCTTCATTCACA		[13]
		QNIFS	6-FAM-AGCACGTGGGAGGGCGATCG-6-TAMRA		[21]
HAV	HAV240		GGAGAGCCCTGGAAGAAAG	174	[5]
	HAV68		TCACCGCCGTTTGCCTAG		[5]
		HAV150	6-FAM-CCTGAACCTGCAGGAATTAA-MGB		[5]
EV	EVR		GGAAACACGGACACCCAAAGTAG	114	[9]
	EVF		TGAATGCGGCTAATCCCAACCTC		[8]
		EVS	6-FAM-TGCGCGTTACGACAGGCCAATCAC-6-TAMRA		[8]
AsV	AV1		CCGAGTAGGATCGAGGGT	90	[14]
	AV2		GCTTCTGATTAAATCAATTTTAA		[14]
		biot-Avs	Biotin- CTTTTCTGTCTCTGTTTAGATTATTTTAATCACC		[14]
RV	VP6.3		GCTTTAAAACGAAGTCTTCAAC	186	[36]
	VP6.4		GGTAAATTACCAATTCCTCCAG		[36]
		biot-RV	Biotin-CAAATGATAGTTACTATGAATGG		[36]
Aichi	6261		ACACTCCCACCTCCCGCCAGTA	342	[37]
	6602		AGGATGGGGTGGATRGGGGCAGAG		[25]
	nested 6309		GTACAAGGACATGCGGCG	160	[25]
	nested 6488		CCTTCGAAGGTCGCGGCRCGGTA		[25]
		biot-Aichi	Biotin-GTACAAGGACATGCGGCG		[25]

been applied to the detection of the main enteric viruses, including NoV [13,18,21], HAV [5], AsV [14], and EV [8]. In this work, this approach was used, together with standard RT-PCR protocols, to monitor the presence of enteric viruses in the Ría de Vigo (Galicia, NW Spain), one of the main bivalve-mollusc-producing areas in the world.

Materials and methods

Molluscs sampling. Sampling was performed monthly, from January to December 2005, in the Ría de Vigo, a large estuary situated in southwestern Galicia (NW Spain), and concurrently with the official sampling program carried out by INTECMAR (Technological Institute for the Marine Monitoring of Galicia). Samples (n = 24) of cultured mussel (*Mytilus galloprovincialis*) were obtained in parallel from two independent floating raft parks. Wild molluscs, including mussel (12 samples), clam (*Ruditapes decussatus*) (3 samples), and cockle (*Cerastoderma edule*) (2 samples), were collected from shore areas close (500–700 m) to the floating rafts. Harvesting areas were classified according to the current EC regulation [Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organization of official controls of the products of animal origin for human consumption. Off J Eur Communities L226:83-127], as B (230–4600 *Escherica coli*/100 g mollusc tissue), for cultured mussels, and C (>4600 *E. coli*/100 g mollusc tissue) for wild molluscs. Each sample consisted of at least 10 mussels or 20 clams/cockles. Molluscs were kept at 4°C and were delivered to the laboratory within 24 h of sampling.

Bivalve processing for virus concentration and RNA extraction. Previous studies demonstrated that most enteric viruses are localized in the stomach and in digestive tissues [29,32], and that sample processing is easier than using the whole mollusc body. Accordingly, on arrival in the laboratory, the molluscs were shucked and their stomach and digestive diverticula dissected. These tissues were then mixed to prepare

1.5-g portions, which were stored frozen (–20°C) until needed. For analysis, the mixed-tissue samples were thawed on ice, homogenized with glycine buffer pH 9.5, and extracted with chloroform-butanol and with Cat-floc (Calgon, Ellwood City, PA, USA). The resulting suspension was then precipitated with polyethylene glycol 6000 (Sigma) [1,16,21]. Viral nucleic acid was extracted and purified from the suspended polyethylene glycol pellet using Nuclisens MiniMAG (BioMérieux, France), a semi-automated extraction procedure involving the use of magnetic particles [18], and then suspended in 100 µl of RNase-free water and kept frozen (–80°C).

Standard RT-PCR. Samples were analyzed for the presence of RV, AsV, and AiV by standard RT-PCR. For AiV, a one-step nested PCR step was necessary to increase the assay's sensitivity. RT was done with a 20-µl mixture containing 2 µl of nucleic acid (NA) extract, 1× buffer II (Applied Biosystems), 5 mM $MgCl_2$, 1 mM dNTP, 2 U of RNase inhibitor (Applied Biosystems), 1.25 µM downstream primer (Table 1), and 50 U of MuLV reverse transcriptase (Applied Biosystems), as previously described [16]. Briefly, after a RT step of 30 min at 42°C (37°C for AiV) and a denaturation step for 5 min at 95°C, PCR mix, containing, at final concentrations, 1× buffer II (Applied Biosystems), 1.25 mM $MgCl_2$, 0.5 µM forward primer (Table 1), and 2.5 U of *Taq* polymerase (Applied Biosystems), was added. These samples were amplified for 40 cycles (94°C for 30 s, 50°C for 30 s, and 72°C for 30 s), with a final extension at 72°C for 7 min, in a thermocycler (9600 or 2400, Applied Biosystems). Nested PCR for AiV was carried out using 2 µl of amplification product and 0.5 µM of each primer (Table 1) under the same PCR conditions as described above. The amplification product was analyzed by PAGE on a 9% polyacrylamide gel, and the product confirmed as a distinct band of adequate intensity after ethidium bromide staining [15].

Samples were considered as positive only if the amplicons were detected by hybridization using specific biotin-labeled probes (Table 1) and the commercial kit Hybridowell Universal (Argene, France), following the manufacturer's instructions. Included in each run were a negative control, containing no nucleic acid, and a specific positive control for each viral pathogen (RNA from viral stocks).

Real-time reverse transcription-PCR. For NoV, HAV, and EV, rRT-PCR was carried out using the Platinum quantitative RT-PCR Thermoscript one-step system (Invitrogen, France). The 25-µl reaction mixture contained 5 µl of extracted RNA, 1× of Thermoscript reaction buffer, 0.9 µM of reverse primer, 0.5 µM of forward primer, 0.45 µM of probe, 0.5 µl of ROX (as a passive internal reference for normalization of the reporter dye signal), and 0.5 µl of Thermoscript Plus/Platinum *Taq* enzyme mix (Invitrogen). Table 1 lists the sequences of primers and probes used. The rRT-PCR was performed with an ABI Prism 7000 SDS detector (Applied Biosystems) or with a Mx3000P QPCR System (Stratagene) in a 96-well format under the following conditions [5,17]: reverse transcription at 55°C for 1 h, denaturation at 95°C for 5 min, followed by 45 cycles of amplification with a denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 65°C for 1 min. Samples showing cycle thresholds (Ct) values ≤41, with no evidence of amplification in the negative controls, were considered as positive.

Extraction and rRT-PCR efficiencies. A mutant non-virulent infective strain of Mengovirus, vMC_0 (kindly provided by A. Bosch, University of Barcelona), was employed as a control for the nucleic acid extraction, as previously described [5]. Prior to the extraction of viral RNA from the mollusc homogenates, the samples were spiked with a known amount (ca. 10^4 plaque-forming units) of vMC_0. Viral RNA extracted from molluscs was tested undiluted and at a ten-fold dilution to evaluate the effect of potential RT-PCR inhibitors. Extraction efficiency values were evaluated by comparing the Ct value for the vMC_0-positive amplification control with that obtained for the tested virus. These results were classified as poor (<1%), acceptable (1–10%), or good (>10%) [7]. To test for the presence of RT-PCR inhibitors and to calculate rRT-PCR efficiency, co-amplifications consisting of 2.5 µl of each extracted RNA and 2.5 µl of a mixture containing 10^3 copies of internal controls for the respective virus type were evaluated in separate experiments [5,17]. The rRT-PCR efficiency was calculated by comparing the Ct value of a sample mixed with internal controls to the Ct value of the internal control alone. Efficiency values were classified in the same three categories as the extraction efficiency (poor, acceptable, and good) [7]. The numbers of viral RNA copies present in positive samples were estimated using standard curves generated from RNA transcripts, as previously described [7,17,18].

Results

Extraction and rRT-PCR efficiencies. The extraction efficiency ranged between 2.3 and 37.9%. According to the classification criteria described above, 28 samples (68.3%) showed good extraction efficiency (>10%) and 13 samples (31.7%) had acceptable extraction values (1–10%). Co-amplifications with internal controls indicated that only partial inhibition was originated by the components of the samples, such that the extracted RNA was considered suitable to test viral presence without false-negative results. Moreover, rRT-PCR efficiencies were always good or acceptable. For NoV GI, 37 of the 41 (90.3%) samples tested showed good rRT-PCR efficiency (>10%) and only four samples (9.8%) had acceptable efficiency (1–10%). For NoV GII, the rRT-PCR efficiency of all samples but one (97.6%) was good.

Viral results. Of the 41 samples examined, 63.4% contained at least one of the viruses studied. In fact, 41.5% of the samples contained one type of virus whereas in 17.1 and 4.8% of the samples, two and three enteric viruses, respectively, were detected. NoV GII was the most prevalent virus, detected in 22 samples (53.7%). In cultured mussels (n = 24), most of the positive samples were infected with only one type of virus (45.8%), although in some samples two or three different types of enteric viruses were detected (4.1% and 8.3%, respectively). In wild molluscs, 12 of the 17 samples contained one (6 samples) or two (6 samples) types of enteric virus (Table 2).

Regarding the mollusc species, six of 12 wild mussels samples contained NoV GII, three samples were positive for EV, two for AsV, and only one sample contained NoV GI. The presence of more than one viral type was detected in five of the wild mussel samples. In addition, all three clam samples were contaminated, one with NoV GII and two with RV, whereas both cockle samples were positive for NoV GII, with one of them also positive for AsV.

Norovirus quantification. The number of viral genomes per gram of mollusc digestive tissue was determined in the NoV-positive samples using the standard curves

Table 2. Number of samples showing the presence of enteric viruses in cultured and wild bivalve molluscs

Virus	Total (n = 41)	Cultured (n = 24)	Wild (n = 17)
NoV GI	1	1	0
NoV GII	13	10	3
HAV	0	0	0
EV	0	0	0
AsV	1	0	1
RV	2	0	2
AiV	0	0	0
NoV GII + EV	3	0	3
NoV GII + NoV GI	2	1	1
NoV GII + AsV	2	0	2
NoV GII + EV + AsV	2	2	0

and taking into account the extraction and rRT-PCR efficiencies. Levels of contamination with both NoV genogroups are shown in Table 3. In general, contamination levels were lower in cultured samples than in wild samples. Three cultured samples rendered positive results but with levels too close to the detection limit of the assay to allow accurate quantification.

For NoV GI, no differences were observed in the levels achieved in cultured and wild samples (<1 log-unit). However, although NoV GII was detected in wild and culture samples at similar highest levels, in 10 out of 13 cultured samples positive for NoV GII, RNA levels were <500 copies/g tissue, whereas in seven out of nine of the wild samples the levels exceeded this value.

Discussion

Current EU regulations establish the use of bacteriological monitoring programs, based on *Escherichia coli* as indicator, to determine the sanitary quality of molluscs and of their harvesting areas [Regulation (EC) No 854/2004]. Several studies have shown that although such controls have been effective at reducing the risk of bacteriological illness to minimal levels, bivalve molluscs meeting the *E. coli* standards may nonetheless contain enteric viruses and therefore act as vectors of human viral diseases [3,19,26,31]. Other proposed indicators of viral contamination, such as F-specific RNA (FRNA) bacteriophages, have been studied with contradictory results [35], highlighting the need for alternative approaches to viral detection.

The purpose of the present study was to test the feasibility of research approaches, specifically, rRT-PCR, in determining the prevalence of the main enteric viruses in cultured and wild molluscs. Samples were collected from the Ría de Vigo (NW Spain), one of the most important European mollusc-harvesting areas, during a one-year period. Despite the importance of this area for mollusc production, few studies have been carried out at this site [30], and none of them included all the enteric viruses analyzed here. The results obtained showed that 63.4% of the samples were contaminated with at least one of the enteric viruses studied. Indeed, this percentage increased to 70.6% if only wild samples were considered. These values are similar to those reported in a similarly contaminated area in France [15].

Few of the previously published studies of viral contamination in molluscs or their harvesting areas [6,19,24,26] included the results of a regular monitoring program [15,22,30]; therefore, little is known about the occurrence of viruses in mollusc beds. In the present study, the most prevalent virus was NoV GII, with EV, AsV, NoV GI, and RV detected at lower frequencies. Similar results were obtained in a 3-year study in France [15] on the prevalence of NoV, EV, and AsV, and in mollusc samples obtained before depuration from a commercial producer in the UK [22]. The decrease in the prevalence of these viruses during warm months is well known [15,16] and can perhaps be explained by the lower circulation levels of these viruses during the summer months [34]. In addition, a more rapid degradation of viral particles at high temperatures and directly by sunlight has been reported [23].

Contrary to the results of a previous study of the same geographic area (Ría de Vigo) [30], HAV was not detected in the samples analyzed. This difference may be due to the different experimental approaches employed, to an improvement in the sanitary conditions of the study area, and/or to a lower endemicity of the viruses in the local population. We also did not detect AiV, recognized as the causative agent in an outbreak of oyster-associated gastroenteritis in Japan in 1989 [37]. This virus was recently detected in oysters implicated in a similar outbreak in France [17]. In our study, wild as well as cultured samples showed evidence of a mixed contamination with more than one enteric virus. This finding is in accordance with the simultaneous detection of different enteric viruses reported in studies performed in other countries [10,19,26,30], usually in bivalve molluscs associated with illness outbreaks. It has been suggested that coinfection with multiple viruses results in more severe disease symptoms [17].

Table 3. Quantification of NoV genogroups I and II in the digestive tissue of bivalve molluscs as determined by rRT-PCR

						RNA copies/g tissue[a]	
NoV genogroup	Sample	Mollusc	% extraction efficiency	% rRT-PCR efficiency	Ct	Uncorrected	Corrected
GI	A20	Mussel	21.1	60.9	39.6	148.6	1155
	C20	Mussel*	25.6	61.8	40.1	101.2	638
	B21	Mussel	14.9	66.7	38.5	327.0	3293
GII	A11	Mussel	18.5	29.7	36.0	75.3	1372
	B11	Mussel	12.9	100	35.3	188.0	1452
	C11	Mussel*	10.4	100	35.3	125.0	1199
	A12	Mussel	14.9	74.1	36.6	49.0	444
	C12	Mussel*	3.4	100	35.4	112.5	3248
	A13b	Mussel	17.5	32.7	41.1	+DL[b]	
	A13m	Mussel	19.8	96.6	41	+DL	
	B13b	Mussel	14.5	22.8	38.1	16.3	495
	C13	Mussel*	19.5	100	33.2	544.6	2787
	A14	Mussel	4.5	15.6	35.5	105.6	15,177
	B14	Mussel	23.9	100	35.7	95.6	400
	C14	Mussel*	17	97.9	37.7	22.4	134
	A18	Mussel	32.1	100	41.5	+DL	
	A19	Mussel	20.8	100	37.5	25.8	124
	B20	Mussel	11.9	66.3	37.8	21.0	266
	C20	Mussel*	25.6	100	36.3	63.6	247
	D20	Clam	7.8	38.7	37.4	28.5	1021
	E20	Cockle	2.9	70	36.9	40.0	2782
	A21	Mussel	35.1	100	37.9	20	56
	B21	Mussel	14.9	100	38.2	16.0	107
	E21	Cockle	2.3	71.1	38.3	14.7	1250
	C22	Mussel*	16.5	100	29.9	5599.7	33,883

[a]Number of RNA copies calculated without taking the extraction and rRT-PCR efficiencies into account (uncorrected) or taking the extraction and rRT-PCR efficiencies into account (corrected).
[b]+DL, positive sample but the level was too low for accurate quantification.
*Wild mussel samples.

In general, and in accordance with data previously reported [30], our results indicate that viral contamination is greater in wild than in cultured bivalves, in this case perhaps due to the proximity of the sampling points to contamination sources. In fact, the wild molluscs analyzed in the present study were harvested at the shoreline, where the urban impact is more evident and the co-occurrence of viral and bacterial pathogens is likely [4,11]. In addition, other factors, including decreased shellfish activity at lower temperatures and the differential retention of viruses by distinct mollusc species, cannot be ruled out [19,26]. Note that, although cultured samples from class B areas have to be depurated before they

can be sold at wholesale or retail markets, since the effectiveness of depuration to eliminate viral contamination is limited [17,20,32], these shellfish can constitute a potential public health hazard.

The quantification of noroviruses in molluscs is a technically complex procedure that is vulnerable to problems such as inhibition of the RT-PCR by mollusc tissue components, which can cause false-negative results [17]. Here, the inclusion as a positive control of a known quantity of an externally added virus that does not interfere in the final results of the quantification, in this case Mengovirus, and of internal specific controls to calculate the extraction and rRT-PCR efficiencies, yielded a more realistic and ultimately successful determination of the viral charge in the mollusc samples. This strategy also allowed us to establish that the assay was not prohibited by mollusc-tissue-mediated inhibition of the RT-PCRs. Interestingly, a recent study by da Silva et al. [6] showed that in some samples only one NoV genogroup was inhibited, implying that inhibitors do not affect the different primers and probes equivalently, although the difference between rRT-PCR efficiencies for GI and GII was not statistically significant. The use of new approaches for quantification overcomes the problems of inhibition and increases the validity of the results, thus allowing better monitoring of shellfish-harvesting areas.

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